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Human monoclonal antibody Ha6D3, a candidate for treatment of leukaemia? *In vitro* reactivity of Ha6D3 with leukaemic cells and *in vivo* applications in a chimpanzee

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Summary The human monoclonal antibody Ha6D3 of the IgM type was used to stain malignant lymphoma cells from peripheral blood in flow cytometry and from cryosections of lymph nodes using the immunoperoxidase technique. It was found to react with peripheral white blood cells of all 12 cases of leukaemia and with lymph node cells of seven out of 11 B cell lymphomas and with the one T cell lymphoma tested so far. For *in vivo* experiments a batch of 70 mg Ha6D3 was purified and 6 mg Ha6D3 was injected intravenously into a chimpanzee with time intervals of 10 months and 1 month. The side effects observed were shivering, some muscular spasms and variations in the heart frequency. A decrease of lymphocytes of more than 50% was documented by haematogram analysis. The flow cytometry data showed that the Ha6D3 antigen does not modulate. Even after three repeated injections applied in a time interval of several months no immune response to Ha6D3 could be detected *in vivo* or *in vitro*. Based on these data we suggest that Ha6D3 may become a candidate for the treatment of certain leukaemias *in vivo*.

Anti-lymphocyte sera are used to treat certain leukaemias although these antisera cross-react with human tissues (Greco *et al.*, 1983). The mouse monoclonal antibody (MAb) OKT3 (anti CD3) which is used for the treatment of graft rejection crises or the rat MAbs CAMPATH-1 (anti CDw52) that have already been used for the treatment of leukaemia *in vivo* elicit immune responses in patients when they are not immunosuppressed (Schroff *et al.*, 1985; Chatenoud *et al.*, 1986; Dyer *et al.*, 1989).

To prevent possible anaphylactic reactions we have produced a human monoclonal antibody (HMAb) named Ha6D3. The reactivity of Ha6D3 against normal cells has been shown to be mainly confined to human B and T cells as could be demonstrated in peripheral white blood cells, lymph nodes and various human tissues (Harpprecht *et al.*, 1988). The antigenic sites detected by this HMAb are located on the outer membrane as well as in intracytoplasmatic structures (Hansmann *et al.*, 1989).

In this study we investigated the reactivity of Ha6D3 against leukaemia cells in peripheral blood by flow cytometry and non-Hodgkin's cells in lymph nodes by immunochemistry. In a chimpanzee model we wanted to answer three questions about the *in vivo* function of Ha6D3. First, can Ha6D3 be given safely; second, will there be an effect on the blood cell populations; and third, will a human monoclonal IgM antibody elicit an immune response in an animal that is as closely related to humans as possible?

Materials and methods

Production and purification of Ha6D3

The production of Ha6D3 has been described before (Harpprecht *et al.*, 1988). Briefly, human spleen lymphocytes were stimulated *in vitro* with irradiated human lymphocytes and fused with the mouse myeloma Ag8. Positive wells were cloned by limiting dilution and the clone Ha6D3 was chosen for further evaluation.

For the *in vivo* applications 10 l of hybridoma supernatants were collected. The cells were grown in culture flasks in serum-free RPMI 1640 supplemented with glutamine, kanamycin and 0.5% HY serum (Gibco). The antibody was precipitated by 90% ammonium sulphate at 4°C, centrifuged and the pellet was dialysed against phosphate buffer containing 1 M sodium chloride. Subsequently Ha6D3 was purified by FPLC gel filtration on a Superose 6 column while the whole FPLC apparatus was kept sterile and endotoxin-free. The mobile phase was phosphate buffered with 1 M sodium chloride. The eluates were pooled and concentrated by ultrafiltration (Amicon XM-300).

The sterility was checked and the bacterial endotoxin content was measured by using a limulus amoebocyte lysate assay (Pyroquant).

Immunohistological staining

For immunohistochemical visualization of antigenic sites the method of Iman *et al.* (1986) was followed. Frozen sections of lymph nodes of 12 cases of malignant lymphoma were incubated with biotinylated HMAb Ha6D3 diluted 1:10 up to 1:100 in Tris-HCl for 30 min. After washing, a complex of avidin-biotin-horseradish peroxidase (Dako) was applied for 30 min, and after a second washing the diaminobenzidine reaction was performed. For control sections Ha6D3 was omitted.

Flow cytometry

Flow cytometry was performed using MAbs against CD3, CD4, CD8 (OKT3, OKT4, OKT11A donated by Ortho Pharmaceutical Co.) and MHC II (B8.12 donated by Dr C. Mawas, Marseille). The reactions were visualized by an FITC-coupled goat antiserum to mouse Ig (Dianova). Staining for Ha6D3 was performed by incubation of cells in Ha6D3 followed by a goat antiserum to human IgM or by using biotinylated Ha6D3 and avidin-FITC. The cells were then fixed with 0.2% formalin and measured in a cell sorter (Becton Dickinson).

Chimpanzee experiments

The chimpanzee used for the experiment was born in the TNO Primate Center and kept in a group under conventional conditions. The injections of Ha6D3 were given under anaesthesia (ketamine) and the animal was kept under anaesthesia for 1 h after the injection. Blood samples were taken at different time intervals.

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Assays for immune responses to Ha6D3

A radioimmunoassay and a cytotoxicity inhibition assay were developed to measure antibody responses to Ha6D3. To produce labelled antibodies for the radioimmunoassay Ha6D3 and, as a control, OKT3-producing cells were cultured in RPMI 1640 containing 100 μCi ml^{-1} ^{3}H-leucine (Amersham). Seventy-five μl of chimpanzee sera or dilutions of them were incubated with 25 μl ^{3}H-labelled antibodies at room temperature for 30 min. One million human lymphocytes were then added to 50 μl of buffer containing 0.01% cycloheximide (Sigma). After 30 min the cells were washed four times and the radioactivity was measured in a β-counter.

For the cytotoxicity inhibition assay Ha6D3 or OKT3 antibodies were diluted in the chimpanzee sera and their reactivity with human lymphocytes was measured in the standard microlymphocytotoxic assay using rabbit complement.

Results

Ha6D3 reactivity with normal and malignant white blood cells

The HMAb Ha6D3 is of the IgM serotype and reacts in cytotoxicity assays, on tissue sections (Harpprecht *et al.*, 1988; Hansmann *et al.*, 1989) and in FACS analysis with virtually all normal human blood lymphocytes.

Flow cytometry of Ha6D3 with peripheral white blood cells obtained from patients with leukaemia also showed reactivity to the leukaemic cells. Eight patients with myeloid leukaemia with only 27 ± 13% CD3 positive cells (normal range in our controls 72 ± 11%) showed 63 ± 23% Ha6D3 positive cells. Three patients with lymphoid leukaemia showed 7 ± 3% CD3 positive *versus* 82 ± 11% Ha6D3 positive cells. A bone marrow aspirate from a patient with acute myeloid leukaemia contained 3% CD3 positive and 43% Ha6D3 positive cells. Two representative cases are demonstrated in Figure 1.

Patient A with acute lymphatic leukaemia showed a severe decrease of CD3 positive cells (8.4%), but 97% of his white blood cells were stained with Ha6D3 (Figure 1a and b). Patient B with acute myeloid leukaemia had 56% CD3 positive cells, whereas 88% of his cells were positive for Ha6D3 (Figure 1c and d).

The HMAb Ha6D3 showed an immunoreaction in frozen sections with most of the malignant non-Hodgkin's lymphomas tested in this series (Table I). It reacted with seven out of

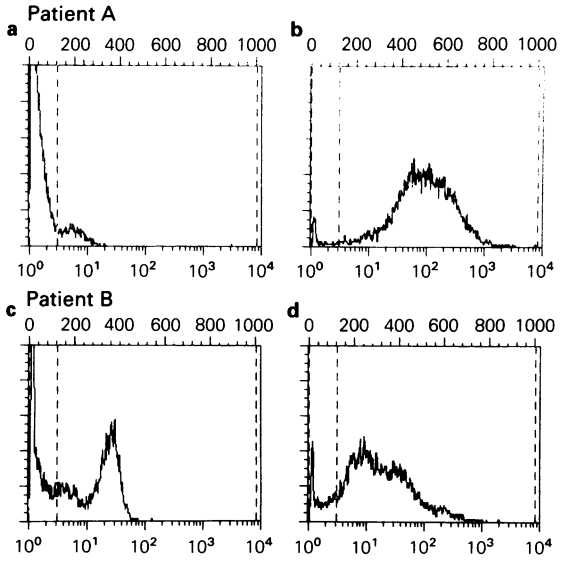

Figure 1 Flow cytometry with peripheral white blood cells. Patient A: acute lymphatic leukaemia, **a** OKT3, **b** Ha6D3. Patient B: acute myeloid leukaemia, **c** OKT3, **d** Ha6D3.

Table I Reactivity of malignant lymphomas of B and T cell types with the HMAb Ha6D3 in frozen sections of lymph nodes

Diagnosis	n *(12)*	*Ha6D3 reactivity*
B cell lymphoma		
chronic lymphatic leukaemia	1	+
centroblastic/centrocytic	4	3 + /1 −
centrocytic	1	+
centroblastic	4	2 + /2 −
immunoblastic	1	−
T cell lymphoma		
large cell anaplastic	1	+

11 malignant lymphomas of B type and with one case of T cell lymphoma. The immunoreaction appears to be localized on the cell membrane of the tumour cells as well as in the cytoplasm (Figure 2a, immunoblastic lymphoma). Sometimes a dot-like intracytoplasmatic immunoreaction could be observed (Figure 2b, centroblastic lymphoma).

In vivo *application of Ha6D3*

To obtain one batch of antibody 10 l of hybridoma supernatant containing about 150 mg Ha6D3 were prepared. Ammonium sulphate precipitation and FPLC gel filtration with a Superose 6 column produced the best results (Figure 3). After purification, sterile filtration and tests for sterility and pyrogen content the total protein yield was 79 mg containing 70 mg Ha6D3 (Table II). A 1 M sodium chloride salt concentration was essential to keep Ha6D3 stable and to prevent the formation of aggregates. The bacterial endotoxin content was 25 USP-EE mg^{-1} Ha6D3. As the limit for humans is 5 USP-EE kg^{-1} body weight, the injection of 6 mg

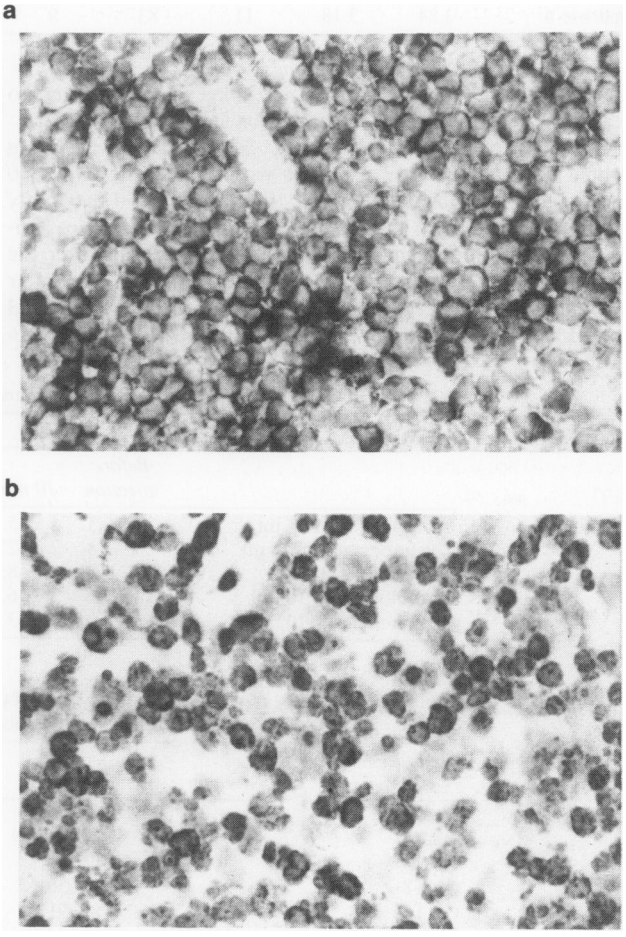

Figure 2 Cryostat section on **a** immunoblastic and **b** centroblastic malignant non-Hodgkin's lymphoma showing a strong immunoreaction with the HMAb Ha6D3. Part of the reaction product seems to be localized in the cytoplasm of the tumour cells. Immunoperoxidase technique, × 350.

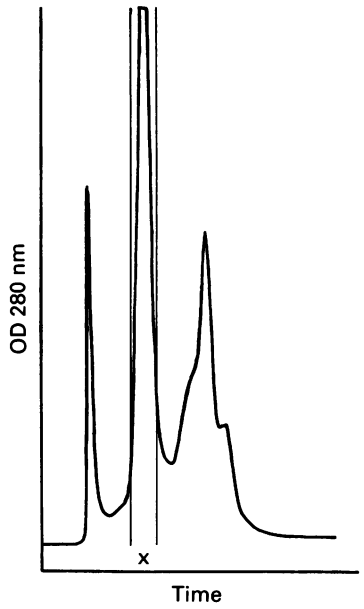

Figure 3 Purification of Ha6D3 with Superose 6. Probes ' × 'containing the Ha6D3 were pooled.

Table II Purification of Ha6D3 by Superose 6 gel filtration. A typical run is shown

	Protein (mg)	*Ha6D3 (mg)*	*Volume (ml)*	*Purity (%)*	*Recovery (%)*
Before Superose 6	33	4.6	1.7	14	—
After Superose 6	3.84	3.18	11	83	9

Ha6D3 into a chimpanzee of about 24 kg was considered to keep the endotoxin level close to this limit.

A female chimpanzee weighing 24 kg was given 6 mg purified Ha6D3 on three occasions with a time interval of 10 months and 1 month between the injections. The body temperature decreased from 37 to 35.5°C during each experiment, possibly due to the anaesthesia. About 10 min after the injections the heart frequency became somewhat irregular and fluctuated between 85 and 105 beats min^{-1}. The animal showed some shivering and muscular spasms. The heart frequency became stable and no shivering or spasms could be detected after about 1 h. The chimpanzee was kept in a single cage for the day of the experiment, but as the overall condition was good she was allowed back to her colony the day after the experiments.

Biochemical analyses showed no changes in liver or kidney functions as the levels of urea, creatinine, alkaline phosphatase, lactate dehydrogenase, glutamic oxalacetic transaminase, glutamic pyruvic transaminase and gamma glutamyl transferase stayed within the normal range.

The haematogram analyses are summarized in Table III. It shows a decrease of lymphocytes from the normal number of 3.1–4.0 × 10^6 cells ml^{-1} to 1.2–2.1 × 10^6 cells ml^{-1} in the time between 1 h and 6 h after all the injections. The number of granulocytes increased from 3.9–5.6 × 10^6 cells ml^{-1} to 19.7–22.2 × 10^6 cells ml^{-1} after 6 h in each experiment. Abnormalities in the cell populations persisted for 1–3 days after the injection, but by day 7 the blood populations were normal again.

The flow cytometry results are summarized in Table IV. They show that in chimpanzees the OKT3 epitope is not present on all T lymphocytes, whereas OKT11A binds to the majority of cells. No significant shifts in the relative number of lymphocyte subsets were observed indicating that no subset was selectively depleted from the circulation. However, lymphocytes that were stained with Ha6D3 *in vivo* could be detected as the number of cells stained only by the FITC-coupled antiserum against human IgM increased from 7–9% before to 21–23% after the injection of Ha6D3, but the amount of antibody injected was too low to see all the lymphocytes clearly labelled. If lymphocytes were stained with additional Ha6D3 *in vitro*, a high percentage of cells could be stained, indicating that the epitope was still present (Table IV, last line).

The radioimmunoassays and the cytotoxicity inhibition assays showed no signs of antibodies against Ha6D3 (Table V). However, a control rabbit antiserum against human IgM inhibited the tests up to a dilution of 1:1000 and the serum of a chimpanzee that had been immunized with OKT3 inhibited OKT3 up to a dilution of 1:100 in these assays. Skin tests with small probes of Ha6D3 did not show any signs of an immune response to the HMAb.

Discussion

The data show that the HMAb Ha6D3 reacted with the white blood cells of 12 cases of leukaemia in flow cytometry

Table III Summary of the haematogram data of the chimpanzee experiments

		Time after the injection					
	Before injection	*10 min*	*1 h*	*6 h*	*1 day*	*3 days*	*7 days*
Erythrocytes × 10^9 ml^{-1}	5.0–5.3	4.6–5.3	4.9–5.4	5.3–5.4	5.2–5.5	5.1–5.2	5.1–5.2
Thrombocytes × 10^8 ml^{-1}	2.2–2.8	2.0–2.9	2.1–2.6	2.7–3.1	2.5–3.2	2.8–3.2	2.4–3.0
Leucocytes × 10^6 ml^{-1}	7.5–9.8	5.9–8.8	4.8–9.9	21.9–26.5	13.1–16.5	11.9–12	6.9–9.7
Granulocytes × 10^6 ml^{-1}	3.6–5.6	3.4–6.5	3.1–7.1	19.7–22.2	9.4–11.7	4.6–6.5	2.8–6.3
Lymphocytes × 10^6 ml^{-1}	3.1–4.0	1.8–3.1	1.2–2.1	2.0–2.6	2.5–3.3	3.9–5.6	4.0–4.4

Table IV Summary of the flow cytometry data of the chimpanzee experiments. The range of the percentage of positively stained lymphocytes is shown

MAb/a-mouse Ig-FITC	*Before*	*10 min*	*1 h*	*6 h*	*1 day*	*2 days*
O	0–1	0–3	0–1	0	0	0
OKT3	47–51	36–69	39–71	29–46	43–44	53–56
OKT4	36–43	47–59	48	44–45	30–42	36–44
OKT8	34–47	20–36	21–31	36–48	43–55	48–49
OKT11.A	82–93	86–91	72–79	86–90	87–93	93–94
B8.12	16–26	7–33	13–29	14–27	11	8–23
HMAb/a-human IgM-FITC						
O	7–9	21–23	11–19	10	0–1	3
Ha6D3	74–82	79–80	67–71	89	76–77	79–80

Table V Radioimmuno and cytotoxicity inhibition assays of the chimpanzee sera for an immune response to Ha6D3 with human cells as targets. A typical experiment is shown

		Time after the injection								
	Before injection	*10 min*	*1 h*	*6 h*	*1 d*	*2 d*	*7 d*	*14 d*	*21 d*	*30 d*
Serum and ^{3}H-Ha6D3 (counts min^{-1})	576	797	816	685	705	880	826	694	717	712
Serum and Ha6D3 (cytotoxic titre)	1/32	1/64	1/128	1/32	1/32	1/32	1/32	1/16	1/16	1/32
	Dilution of rabbit anti-human IgM in normal chimpanzee serum									
Controls	*1:100*	*1:1000*	*1:10000*							
^{3}H-Ha6D3 (counts min^{-1})	179	119	463							
Ha6D3 (cytotoxic titre)	None	None	1:8							
	Dilution of a chimpanzee serum immunized with OKT3 in normal chimpanzee serum									
	1:5	*1:25*	*1:100*	*No antiserum*						
^{3}H-OKT3 (counts min^{-1})	32	34	31	135						
OKT3 (cytotoxic titre)	None	None	1:128	1:512						

and with cryostat sections of seven out of 11 B cell lymphomas and with one T cell lymphoma tested by the immunoperoxidase technique.

To find out whether Ha6D3 can be administered safely, whether there will be an effect on the lymphocyte populations, and whether Ha6D3 elicits an immune response, an *in vivo* experiment was performed. A batch of Ha6D3 was produced and 70 mg were obtained after purification, representing a yield of about 50%. This shows that using the methods described, a sufficient batch of highly purified Ha6D3 for *in vivo* experiments could be produced even on a laboratory scale.

Three injections of 6 mg Ha6D3 were administered at time intervals longer than 1 month and no severe side effects were observed. Haematogram analyses from blood taken before and after the injections of Ha6D3 showed a 50% decrease of all the lymphocytes and a remarkable increase of granulocytes. It is unlikely that *in vivo* a single injection of 6 mg Ha6D3 would have destroyed lymphocytes in all compartments of the haematoipoietic system, so that these lymphocytes could be released into the peripheral blood.

The flow cytometry data indicate that the Ha6D3 epitope was still present all the time after the injection. When cells were already weakly stained *in vivo* extra Ha6D3 could stain them even more *in vitro*, indicating that the Ha6D3 epitope was not modulated from the cell surface during the experiments. Therefore, in contrast to experiments with the MAb T101 (Bertram *et al.*, 1986), a second injection of Ha6D3 given 1 day later will be expected to be effective.

Even after adequate time intervals to allow the possible generation of an immune response, no inhibiting antibodies could be detected *in vivo* or *in vitro*. In contrast, when MAbs of mouse or rat origin were injected in clinical trials severe immune responses in some patients made further injections of the MAbs impossible (Bertram *et al.*, 1986; Dyer *et al.*, 1989). On the other hand, first trials with a genetically reshaped human IgG_1 CAMPATH-1 antibody gave promising results without any signs of an immune response (Waldmann, personal communication; Hale *et al.*, 1988).

In summary, we suggest that Ha6D3 may become a candidate for a pilot study in the treatment of leukaemia patients who do not respond to conventional chemotherapy.

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